P53-REGULATED GENES

TECHNICAL FIELD OF THE INVENTION

The invention relates to the area of gene regulation, in particular the area of regulation of genes involved in tumorigenesis.

BACKGROUND OF THE INVENTION

P53 is the name of a human tumor suppressor gene and its protein product. About 55 percent of all human cancers from many cell or tissue types suffer mutations in both alleles of the p53 gene. People who have inherited such mutations, will develop cancer over their lifetimes.

The p53 protein is a transcription factor, which regulates the expression of a large number of genes. High levels of an active wild type p53 protein in a cell cause these genes to be transcribed at a high rate. Elucidation of the functions of some of these "p53-regulated or -inducible genes" informs the art of how the p53 protein protects humans from cancers.

There is a need in the art to discover p53-inducible genes and their functions, so that we may have a chance to circumvent the effects of p53 mutations in cancer, by activating the p53-inducible genes and repressing the p53-repressible genes, so as to arrest the cancer's growth. Thus, the elucidation of such p53-regulated genes provides useful and valuable information.

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SUMMARY OF THE INVENTION

In one embodiment, a method is provided for diagnosing cancer or for determining p53 status in a sample suspected of being neoplastic. The level of expression of an RNA transcript or its translation product in a first sample of a first tissue is compared to the level of expression of the transcript or translation product in a second sample of a second tissue. The first tissue is suspected of being neoplastic and the second tissue is a normal human tissue. The first and second tissue are of the same tissue type. The transcript is a transcript of a gene selected from the group consisting of gene numbers 1-8, 10, 12, 14-58, 60-68, and 70-100, as shown in Figure 1. The first sample is categorized as neoplastic or as having a mutant p53 when expression is found to be the same or lower in the first sample than in the second sample.

According to another embodiment a method is provided for diagnosing cancer or for determining p53 status in a sample suspected of being neoplastic. The level of expression of an RNA transcript or its translation product in a first sample of a first tissue is compared to the level of expression of the transcript or translation product in a second sample of a second tissue. The first tissue is suspected of being neoplastic and the second tissue is a normal human tissue. The first and second tissue are of the same tissue type. The transcript is a transcript of a gene selected from the group consisting of gene numbers 7-24, and 26-100 as shown in Figure 2. The first sample is categorized as neoplastic or as having a mutant p53 when expression is found to be the same or higher in the first sample than in the second sample.

Another aspect of the invention is a method of diagnosing cancer or determining p53 status in a sample suspected of being neoplastic. The level of expression of at least one RNA transcript or its translation product in a first sample of a first tissue is compared to the level of expression of the transcripts or translation products in a second sample of a second tissue. The first tissue is suspected of being neoplastic and the second tissue is a normal human tissue. The first and second tissue are of the same tissue type. The first group of RNA transcripts consists of transcripts of genes selected from the group of genes numbered 1-8, 10, 12, 14-58, 60-68, and 70-100 as shown in Figure 1. The second group of RNA transcripts consists of

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transcripts of genes selected from the group consisting of genes numbered 7-24, and 25-100 as shown in Figure 2. The first sample is categorized as neoplastic or as having a mutant p53 when expression of at least one of the first group of RNA transcripts or translation products is found to be the same or lower in the first sample than in the second sample, and expression of at least one of the second group of transcripts or translation products is found to be the same or higher in the first sample than in the second sample.

According to another aspect of the invention a method is provided for evaluating carcinogenicity of an agent. A test agent is contacted with a human cell. The level of expression of at least one transcript or its translation product is determined in the human cell after contacting with the agent. The transcript is of a gene selected from the group consisting of genes numbered 1-8, 10, 12, 14-58, 60-68, and 70-100 in Figure 1 and genes numbered 7-24, and 26-100 in Figure 2. An agent which decreases the level of expression of a gene identified in Figure 1, or an agent which increases the level of expression of a gene identified in Figure 2 is identified as a potential carcinogen.

Another aspect of the invention is directed to a method of treating cancer in a patient. A polynucleotide is administered to cancer cells of a patient. The polynucleotide comprises a coding sequence of a gene selected from the group consisting of genes numbered 1-8, 10, 12, 14-58, 60-68, and 70-100 in Figure 1. The cancer cells of the patient harbor a mutant p53 gene. As a result of the administration, the gene is expressed in cells of the cancer.

Yet another aspect of the invention is directed to a method of treating cancer in a patient. An antisense construct comprising at least 12 nucleotides of a coding sequence of a gene selected from the group consisting of genes numbered 7-24, and 26-100 in Figure 2 is administered to cancer cells of a patient. The coding sequence is in 3' to 5' orientation with respect to a promoter which controls its expression. The cancer cells harbor a mutant p53 gene. As a result of the administration, an antisense RNA is expressed in cells of the cancer.

According to still another aspect of the invention a method is provided of screening for drugs useful in the treatment of cancer. A cell which harbors a p53

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mutation is contacted with a test substance. Expression of a transcript or its translation product is monitored. The transcript is of a gene selected from the group consisting of genes numbered 1-8, 10, 12, 14-58, 60-68, and 70-100 in Figure 1 and genes numbered 7-24, and 26-100 in Figure 2. A test substance is identified as a potential drug for treating cancer if it increases expression of a gene as shown in Figure 1 or decreases expression of a gene as shown in Figure 2.

Another aspect of the invention is a method of screening for drugs useful in the treatment of cancer. A tumor cell which overexpresses MDM2 is contacted with a test substance. Expression of a transcript or its translation product is monitored. The transcript is of a gene selected from the group consisting of genes numbered 1-8, 10, 12, 14-58, 60-68, and 70-100 in Figure 1 and genes numbered 7-24, and 26-100 in Figure 2. A test substance is identified as a potential drug for treating cancer if it increases expression of a gene as shown in Figure 1 or decreases expression of a gene as shown in Figure 2.

Yet another aspect of the invention provides a set of at least two nucleotide probes which hybridize to a set of p53-regulated genes. The genes are selected from the group consisting of genes numbered 1-8, 10, 12, 14-58, 60-68, and 70-100 in Figure 1 and genes numbered 7-24, and 26-100 in Figure 2.

These and other embodiments of the invention provide the art with methods for diagnosis, treatment and drug discovery for cancers. In addition, it provides a convenient and rapid carcinogenicity test.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a Table showing genes induced by p53. The column headings are as follows. "Eb1" denotes the cell line EB1-α , *i.e.,* a cell line which contains a zinc-inducible p53 gene. "EB" denotes the cell line EB1, a cell line which has a p53 mutant gene that fails to produce or express detectable p53 protein. "PM" denotes the number of perfect match oligonucleotides for a gene which hybridized and "MM" denotes the number of mismatch oligonucleotides for a gene which hybridized. "Ratio" is the ratio of intensity of EB1-α to EB1. "Accession number" refers to a GenBank accession number. "EST?" if checked indicates that the function of the nucleic acid sequence has not been determined. "SAGE?" if checked indicates that analysis using the SAGE

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technique also detected this gene as p53-regulated. See http://welchlink.welch.jhu.edu/~molgen-g/P53-SAGE.HTM.

Figure 2 is a Table showing genes repressed by p53. Column headings are the same as in Figure 1.

DETAILED DESCRIPTION

It is a discovery of the present inventors that p53 regulates a whole host of genes, increasing and decreasing expression of their mRNA and protein products. These genes have not previously been identified as p53 regulated. In some cases the biological functions of the genes is unknown. However, the now-established regulation by p53 indicates that the genes are involved in progression and arrest of the cell cycle.

A sampling of 6800 human genes were tested for the effects of p53 expression on their expression. Such a massive screening permits the identification of many genes which were heretofore not known to be p53 regulated.

Genetic status of p53 alleles (mutant or wild-type) has been shown to correlate well with a neoplastic state. Thus diagnosis can be provided based on the status of p53 alleles of cells. The level of expression of an RNA transcript or its translation product can be determined using any techniques known in the art. Specific oligonucleotide probes for the relevant genes can be used in hybridization experiments, as is known in the art. Any hybridization format for determining specific RNA levels can be used, including but not limited to Northern blots, slot blots, dot blots, and hybridization to oligonucleotide arrays. Specificity of hybridization can be assessed by varying degrees of stringency of the hybridization conditions. In addition, comparison of mismatch to perfect match oligonucleotide probes can be used to determine specificity of binding. To assess specific translation product (protein) expression levels, antibodies specific for the protein can be used readily. Again, any format known in the art for measuring specific protein levels can be used, including sandwich assays, ELISAs, immunoprecipitations, and Western blots. Any of monoclonal antibodies, polyclonal antibodies, single chain antibodies, and antibody fragments may be used in such assays. Specificity of immunologic reactions can be assessed using competitor antibodies or

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proteins, as well as by varying the immunoreaction conditions. Monitoring expression product levels involves determining amounts of a specific expression product. Amounts determined need not be absolute amounts, but may be relative amounts determined under different conditions, for example, in the presence and absence of a

5   test compound.

Probes according to the present invention may be labeled or unlabeled, tethered to another substance or in solution, synthetically made or isolated from nature. Probes can be nucleic acids, either RNA or DNA, which contain naturally occurring nucleotide bases or modified bases. The probes may contain normal nucleotide bonds or peptide

10   bonds. Oligonucleotide probes may be of any length which provides meaningful specificity of hybridization. Thus probes may be as small as 10 nucleotides, and preferably they are between 12 and 30 nucleotides in length. However, oligonucleotide probes may be significantly longer, in the range of 30 to 100 nucleotides, 100 to 500 nucleotides or 500 to 2000 nucleotides. Probes may be attached to polymers, either

15   soluble or non-soluble. Probes may be attached or bonded to solid substrates such as filters, sheets, chips, slides, and beads.

High density arrays are particularly useful for monitoring the expression control at the transcriptional, RNA processing and degradation level. The fabrication and application of high density arrays in gene expression monitoring have been

20   disclosed previously in, for example, WO 97/10365, WO 92/10588, U.S. Application Ser. No. 08/772,376 filed December 23, 1996; serial number 08/529,115 filed on September 15, 1995; serial number 08/168,904 filed December 15, 1993; serial number 07/624,114 filed on December 6, 1990, serial number 07/362,901 filed June 7, 1990, all incorporated herein for all purposed by reference. In some embodiments using high

25   density arrays, high density oligonucleotide arrays are synthesized using methods such as the Very Large Scale Immobilized Polymer Synthesis (VLSIPS) disclosed in U.S. Pat. No. 5,445,934 incorporated herein for all purposes by reference.   Each oligonucleotide occupies a known location on a substrate. A nucleic acid target sample is hybridized with a high density array of oligonucleotides and then the amount

30   of target nucleic acids hybridized to each probe in the array is quantified.   One preferred quantifying method is to use confocal microscope and fluorescent labels.

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The GeneChip® system (Affymetrix, Santa Clara, CA) is particularly suitable for quantifying the hybridization; however, it will be apparent to those of skill in the art that any similar systems or other effectively equivalent detection methods can also be used.

Tissue samples which can be tested according to the present invention are any which are derived from a patient, whether human, other domestic animal, or veterinary animal. Vertebrate animals are preferred, such as mice, humans, horses, cows, dogs, and cats, although any organism for which p53 status can be determined may be used. The form of the samples may be any which are routinely used in the art for determining the amounts of specific proteins or mRNA molecules. The samples may be fixed or unfixed, homogenized, lysed, cryopreserved, etc. It is most desirable that matched tissue samples be used as controls. Thus, for example, a suspected colorectal cancer tissue will be compared to a normal colorectal epithelial tissue.

Figures 1 and 2 below show genes which are induced by p53 and repressed by p53, respectively. Genes which are identified with a check in the column headed "SAGE" are those which are believed to be previously identified as p53-regulated. Genes which are not checked are believed to be previously unknown as p53-regulated genes. Genes numbered 1-8, 10, 12, 14-58, 60-68, and 70-100 in Figure 1, and genes numbered 7-24, and 26-100 in Figure 2, are believed to be such previously unknown p53-regulated genes.

While assaying expression of any single one of the genes identified as p53-regulated may be useful for diagnosis and assays, it may be desirable to use larger sets to confirm the global cellular effects observed. In this regard, it may be desirable to assay for at least 2, 5, 10, 20, 30, 32, 50, 70, or 77 genes of one or both categories of regulation. It may be useful to use both induced and repressed genes to get such a global snapshot of gene regulation.

In a particularly preferred embodiment, oligonucleotide probes for RNA transcripts are attached to solid supports. Such supports are preferably arrays where nucleic acid molecules are attached to the substrate in predetermined positions. In one particular embodiment, the nucleic acid molecules are synthesized on the substrate. In

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another embodiment the nucleic acid molecules are applied to the solid support after synthesis or isolation.

Test samples for mRNA are typically harvested from the tissue samples and may be used directly or processed as follows. The sample mRNA is reverse transcribed using reverse transcriptase to form cDNA. A promoter is ligated to the cDNA at its 5', 3', or both ends. (5' and 3' refer to orientation on the coding strand of DNA.) If two promoters are used on one cDNA they can be the same or different. The cDNA is then used as a template to transcribe *in vitro* to form test mRNA. The test RNA can then be used to hybridize to nucleic acid molecules or probes, preferably on a solid support, more preferably on an oligonucleotide array. These processing steps are well known in the art.

The regulated genes discovered here can form the basis of a carcinogenicity test. Test agents are evaluated to see if their effects on human cells mimic the effects of loss of p53. Thus the agents are in essence being evaluated for the ability to induce a p53 mutation, or a mutation in another gene which is in the same regulatory pathway, or a non-genetic effect which mimics p53 loss. Test agents which are found to have at least some of the same constellation of effects as p53 loss on the regulation of the genes identified herein to be p53-regulated, are identified as potential carcinogens. Any single gene identified can be used, as can at least 2, 5, 10, 20, 30, 32, 40, 50, 70, 90, 100, 125, or 145 of the genes identified herein.

The genes identified herein as p53-induced can be delivered therapeutically to cancer cells. Antisense constructs of the genes identified herein as p53-repressed can be delivered therapeutically to cancer cells. The goal of such therapy is to retard the growth rate of the cancer cells. Expression of the sense molecules and their translation products or expression of the antisense mRNA molecules has the effect of inhibiting the growth rate of cancer cells or inducing apoptosis (a radical reduction in the growth rate of a cell). Sense nucleic acid molecules are preferably delivered in constructs wherein a promoter is operatively linked to the coding sequence at the 5'-end and initiates transcription of the coding sequence. Anti-sense constructs contain a promoter operatively linked to the coding sequence at the 3'-end such that upon

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initiation of transcription at the promoter an RNA molecule is transcribed which is the complementary strand from the native mRNA molecule of the gene.

Delivery of nucleic acid molecules can be accomplished by many means known in the art. Gene delivery vehicles (GDVs) are available for delivery of polynucleotides to cells, tissue, or to a the mammal for expression. For example, a polynucleotide sequence of the invention can be administered either locally or systemically in a GDV. These constructs can utilize viral or non-viral vector approaches in in vivo or ex vivo modality. Expression of such coding sequence can be induced using endogenous mammalian or heterologous promoters. Expression of the coding sequence in vivo can be either constitutive or regulated. The invention includes gene delivery vehicles capable of expressing the contemplated polynucleotides. The gene delivery vehicle is preferably a viral vector and, more preferably, a retroviral, adenoviral, adeno-associated viral (AAV), herpes viral, or alphavirus vectors. The viral vector can also be an astrovirus, coronavirus, orthomyxovirus, papovavirus, paramyxovirus, parvovirus, picornavirus, poxvirus, togavirus viral vector. See generally, Jolly, Cancer Gene Therapy 1:51-64 (1994); Kimura, Human Gene Therapy 5:845-852 (1994), Connelly, Human Gene Therapy 6:185-193 (1995), and Kaplitt, Nature Genetics 6:148-153 (1994).

Delivery of the gene therapy constructs of this invention into cells is not limited to the above mentioned viral vectors. Other delivery methods and media may be employed such as, for example, nucleic acid expression vectors, polycationic condensed DNA linked or unlinked to killed adenovirus alone, for example see Curiel, Hum Gene Ther 3:147-154 (1992) ligand linked DNA, for example, see Wu, J. Biol. Chem. 264:16985-16987 (1989), eucaryotic cell delivery vehicles cells, for example see U.S. Serial No. 08/240,030, filed May 9, 1994, and U.S. Serial No. 08/404,796, deposition of photopolymerized hydrogel materials, hand-held gene transfer particle gun, as described in U.S. Patent No. 5,149,655, ionizing radiation as described in U.S. Patent No. 5,206,152 and in PCT Patent Publication No. WO 92/11033, nucleic charge neutralization or fusion with cell membranes. Additional approaches are described in Philip, Mol. Cell. Biol. 14:2411-2418 (1994) and in Woffendin, Proc. Natl. Acad. Sci. 91:1581-585 (1994). Particle mediated gene transfer may be

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employed, for example see U.S. provisional application No. 60/023,867. Briefly, the sequence can be inserted into conventional vectors that contain conventional control sequences for high level expression, and then be incubated with synthetic gene transfer molecules such as polymeric DNA-binding cations like polylysine, protamine, and albumin, linked to cell targeting ligands such as asialoorosomucoid, as described in Wu and Wu, J. Biol. Chem. 262:4429-4432 (1987), insulin as described in Hucked, Biochem. Pharmacol. 40:253-263 (1990), galactose as described in Plank, Bioconjugate Chem 3:533-539 (1992), lactose or transferrin. Naked DNA may also be employed. Exemplary naked DNA introduction methods are described in PCT Patent Publication No. WO 90/11092 and U.S. Patent No. 5,580,859. Uptake efficiency may be improved using biodegradable latex beads. DNA coated latex beads are efficiently transported into cells after endocytosis initiation by the beads. The method may be improved further by treatment of the beads to increase hydrophobicity and thereby facilitate disruption of the endosome and release of the DNA into the cytoplasm. Liposomes that can act as gene delivery vehicles are described in U.S. Patent No. 5,422,120, PCT Patent Publication Nos. WO 95/13796, WO 94/23697, and WO 91/144445, and EP No. 524,968.

Drugs useful in the treatment of cancer can be screened and identified using the p53-regulated genes disclosed herein. Cells of two types can be contacted with test substances. The cells may not carry a wild-type p53 allele or the cells may overexpress the MDM2 gene product. MDM2 sequesters wild-type p53. Therefore MDM2-overexpressing cells mimic cells which are genetically deficient for p53. Expression of one or more of the p53-regulated genes of the present invention is monitored in the presence of the test substance. A test substance which mimics one or more of the regulatory effects of p53 on the p53-regulated genes is a potential therapeutic agent for treating cancer. Such agents can be subsequently tested in any number of other assays to determine their ultimate usefulness as a drug.

Sets of at least two oligonucleotide probes are provided. Preferably the probes are exclusively perfectly matched probes to the genes. However, mismatch probes having less than 5% mismatched nucleotides can be used. The probes hybridize to the p53-regulated genes disclosed herein in Figures 1 and 2. Particularly useful genes are

those numbered 1-8, 10, 12, 14-58, 60-68, and 70-100 in Figure 1, and those numbered 7-24, and 26-100 in Figure 2. It is preferred that all of the oligonucleotide probes hybridize to p53-regulated genes, although it is permissible to have probes for other genes present which are not p53 regulated. Preferably the probes for other genes comprise less than 50% of the probes, and more preferably comprise less than 25%, 15%, 10%, 5%, 2%, or 1%. The sets of p53 regulated genes may comprise at least five, ten, fifteen, twenty, twenty-five, thirty, fifty, seventy-five, 100, or 140 oligonucleotide probes p53-regulated genes, as disclosed herein. The probes may be attached to a polymer, soluble or insoluble, naturally occurring or synthetic. The probes may be attached to a solid support, in a gel matrix, or in solution. The probes may be individually packaged and contained within a single container, or may be mixed in one or more mixtures. Preferably the probes are arrayed on a solid support.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only, and are not intended to limit the scope of the invention. The following methods were used in the examples reported below.

EXAMPLE

Eb-1 cells were derived from a human colon carcinoma and these cells have a p53 mutant gene that fails to produce or express detectable p53 protein. A p53 wild-type gene was inserted into these cells under the regulation of a metallothionein (MT) promoter. In the presence of zinc, this promoter expresses the p53 gene but in the absence (or low levels) of zinc, little or no p53 mRNA or protein are produced. Thus, p53 mRNA and protein are zinc-inducible and the p53-regulated genes are similarly induced by the addition of zinc. The Eb-1 cells (original cell line) without a p53 inducible wild-type gene are called Eb-1 and Eb-1 cells with the MT-p53 inducible gene are termed Eb-1 (α).

Eb-1 cells and Eb-1 (alpha) cells were grown in culture and either left untreated or exposed to zinc. Four hours and ten hours after the addition of zinc, these cells were harvested for analysis.

The messenger RNA was extracted from these cells and purified. An oligo-dT primer was used to produce a reverse transcriptase copy of the mRNA and then, an oligonucleotide linker was ligated to the end of the cDNA where the linker has a T-7 promoter sequence incorporated into it. All of the cDNAs were cloned into vectors, which were then employed to make mRNA copies *in vitro* using the T-7 RNA polymerase and fluorescent biotinylated nucleotides. The RNA products were hydrolyzed to an average size of 50 nucleotides in length.

The hydrolyzed RNA was hybridized to a chip that contains deoxyoligonucleotide sequence (25 in length) that derive from a database of 6,800 known genes or EST sequences. There is a 20-fold redundancy for each gene or EST sequence and for each perfect sequence match, a mismatched sequence (one base different in the middle of the sequence of 25 nucleotides).

After a short hybridization, that measures the rate (amount) of fluorescent probe hybridized to each set of 20 oligonucleotide sequences, the chips are washed and read by a digital confocal microscope to quantitate the intensity of the fluorescent readout. Gene expression or mRNA concentration is measured by changes in the fluorescent readout for probe pairs. The specificity of the measurements is given by the ratio of hybridization to a perfect sequence matched probe compared with the hybridization to a mismatched probe.

For this experiment a control was run; Eb-1 cells treated or untreated with zinc. Here no p53 cDNA was present so the only variable was the exposure of the cells to zinc. Out of all the genes or sequences tested in these cells (6,800), only six changed their gene expression patterns in response to added zinc and five (1-5) of these six genes are under the control of zinc and cadmium inducible promoters. This experiment serves as an excellent control for the p53 regulation of genes.

For the experiment, Eb-1 (α) cells were treated with zinc or left untreated and at four or ten hours, the cells were harvested. RNA was prepared and processed as described above. The hybridization to the 6,800 different oligonucleotide sequences on the chip (each cDNA had a twenty-fold sequence redundancy covering different

5 sequences in the cDNA) was carried out and the analysis of the data was done by an algorithm. The following criteria were employed to accept a gene as p53-inducible or repressed by p53: (1) the relative intensity of the mRNA hybridization level of an induced gene or a decrease with a repressed gene, was above 160 relative units; this has been shown to be a reproducible level with minimal statistical fluctuations; (2) the

10 fraction of probe pairs (matched hybridized and mismatch not hybridized) had to be 0.85 or greater (17 out of 20 perfect matches); (3) the ratio of induction by p53 or repression by p53, when comparing cellular RNA from Eb-1 zinc-induced cells, was five-fold or greater. Thus, only genes whose mRNA levels increased five-fold or decreased five-fold from a high baseline are reported by this analysis.

15 Of 6,800 cDNAs detectable on the chip, 70 genes were induced by p53 and 77 were repressed by p53. Once again, there were excellent positive controls in this experiment. The known p53 inducible genes such as p21-WAF-1, IGF-BP-3, MDM-2, GAD-45, as well as some recently reported 53 inducible genes (PIGS) were detected by this chip hybridization. Similarly, a repressed gene, MAP-4 previously reported in

20 the literature was repressed after p53 induction in Eb-1(α) cells as detected in this experiment.

Figure 1 lists the genes which are induced by p53 and Figure 2 lists the genes which are repressed by p53.